

US 9,810,471 B2

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the GLUTX gene and detect mutations by comparing the sequence of the sample GLUTX with the

5 corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxim and Gilbert (*Proc. Natl. Acad. Sci. USA* 74:560 (1977)) or Sanger (*Proc. Natl. Acad. Sci. USA* 74:5463). It is also contemplated that any of a variety of

10 automated sequencing procedures can be utilized when performing the diagnostic assays (*Bio/Techniques* 19:448, 1995) including sequencing by mass spectrometry (see, e.g. PCT International Publication No. WO 94/16101; Cohen *et al.*, *Adv. Chromatogr.* 36:127-162 1996; and Griffin *et al.*, *Appl. Biochem. Biotechnol.* 38:147-159, 1993).

Other methods of detecting mutations in the GLUTX gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.* *Science* 230:1242 1985).

20 In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes formed by hybridizing (labeled) RNA or DNA containing the wild-type GLUTX sequence with potentially mutant RNA or DNA obtained from a tissue sample.

The double-stranded duplexes are treated with an agent

25 which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digest the mismatched regions.

30 In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting

material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation.

(see, for example, Cotton et al., *Proc. Natl. Acad. Sci. USA* 85:4397 1988; Saleeba et al., *Methods Enzymol.* 217:286-295 1992). In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in GLUTX cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches (Hsu et al., *Carcinogenesis* 15:1657-1662 1994). According to an exemplary embodiment, a probe based on a GLUTX sequence is hybridized to a cDNA or other DNA product from a test cell or cells. The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. See, for example, U.S. Patent No. 5,459,039.

In other embodiments, alterations in electrophoretic mobility can be used to identify mutations in GLUTX genes. For example, single strand conformation polymorphism (SSCP) can be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita et al., *Proc. Natl. Acad. Sci. USA* 86:2766, see also Cotton *Mutat Res.* 285:125-144 1993; and Hayashi *Genet. Anal. Tech. Appl.* 9:73-79 1992). Single-stranded DNA fragments of sample and control GLUTX nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments

may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred
5 embodiment, the method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Kee *et al.*, *Trends Genet.* 7:5 1991).

In yet another embodiment, the movement of mutant or
10 wild-type fragments in a polyacrylamide gel containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE; Myers *et al.*, *Nature* 313:495, 1985). When DGGE is used as the method of analysis, DNA will be modified to insure that it does not completely
15 denture, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum *et al.*, *Biophys. Chem.*
20 265:12753, 1987).

Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide
25 primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki *et al.*, *Nature* 324:163, 1986); Saiki *et al.*, *Proc. Natl. Acad. Sci. USA* 86:6230, 1989). Such
30 allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.